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Purinergic receptors in the carotid body as a novel target for controlling hypertension

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Abstract

Given the proportion of individuals with resistance to, and poor compliance or tolerance of, anti-hypertensive medication new drugs to treat this syndrome are required urgently. We show that peripheral chemoreceptors generate aberrant signalling contributing to high blood pressure in hypertension and thus reveal a novel target. We discovered that *P2x3* receptor mRNA expression was up regulated substantially in chemoreceptive petrosal sensory neurones in hypertensive rats. These neurones generated both tonic drive and hyperreflexia in hypertensive (but not normotensive rats), and both phenomena were normalised by blockade of P2X3 receptors. Antagonism of P2X3 receptors also reduced arterial pressure and basal sympathetic activity and normalised carotid body hyperreflexia in conscious hypertensive rats; no effect was observed in normotensive rats. These preclinical data support the P2X3 receptor as a putative novel target for controlling human hypertension, a notion supported by our evidence of both P2X3 receptor expression in hypertensive human carotid body and the revelation of its hyperactivity.

Introduction

One third of the human population is hypertensive. In the USA, despite 75% of individuals using anti-hypertensive medication only 53% (~41 million) have blood pressure controlled[1]. This may, in part, reflect the significant drug adherence problem due to the asymptomatic nature of hypertension and the poorly tolerated side-effects of current medication. Significant savings in healthcare costs are predicted if blood pressure was reduced to <140/90 mm Hg[2]. The clear evidence that a 10 mmHg rise in diastolic BP >115/75 mm Hg *doubles* the risk of death from cardiovascular disease in adults over 40 years[3]. Further, at least 8% of an estimated 900 million hypertensive humans worldwide[4] currently have developed drug-resistance[5,6]. Categorically, there remains an unmet clinical need for controlling blood pressure.

Aliskiren, a renin inhibitor, was the last anti-hypertensive drug introduced in 2007. This was the first new drug for thirteen years, yet its efficacy beyond the existing renin-angiotensin–aldosterone enzyme inhibitors and receptor blockers is questionable, and associated adverse effects question its long-term benefit-harm relationship[7]. The paucity of novel pharmacological anti-hypertensives has led to a series of device based interventional studies including renal denervation[8], stimulation of carotid baroreceptors[9], deep brain stimulation[10] and arterial venous anastomosis[11,12]. Here, we introduce a novel mechanistic approach using a highly selective small molecule antagonist, and provide the pre-clinical and clinical data that justifies its use in a future clinical trial in hypertension.

Currently the carotid body is being considered as a novel therapeutic target for cardiovascular disease: (i) the carotid body chemoreflex-evoked sympathoexcitatory

response is potentiated in both a pre-clinical model of hypertension - the spontaneously hypertensive (SH) rat - and humans with hypertension[13-18]; (ii) denervation of the carotid body is evidently an effective way to control both the development and maintenance of high blood pressure in the SH rat[19,20]. We concluded that in conditions of hypertension the carotid body chemoreceptors generate aberrant excitatory tone driving up sympathetic activity and causing high blood pressure[14,21]. We found that by switching off this carotid body tone using either hyperoxia or nerve section to disconnect the carotid body from the brain, arterial pressure and sympathetic activity were reduced in SH rats, but were unaffected in a normotensive strain[14]. In hypertensive human, hyperoxia lowered sympathetic activity[18] and carotid body resection can reduce arterial pressure[22]. Thus, the animal and human data support the presence of aberrant carotid body discharge contributing to hypertension. Although the mechanistic basis for the carotid body aberrant tone is unknown, it may be pivotal for refining new pharmacological anti-hypertensive approaches as we propose here.

It is acknowledged that a plethora of mechanisms governs carotid body signalling[23-27]. We have considered in our current investigations the ATP gated ion channels (called purinergic P2X receptors), specifically the C-fiber localised P2X3 receptor subtypes, which are commonly associated with afferent sensitisation, and that apparently may contribute to hyperreflexic disease states in a variety of organs[28,29]. ATP is one of a number of transmitters involved in the transduction process of hypoxia in the carotid body[30-35]. P2X3 receptor expression is present in both carotid bodies and petrosal ganglia neurones of normotensive rats[36]. Moreover, combined P2X2 and P2X3 subunit deletion reduced the ventilatory response to hypoxia in mice[37]. We hypothesised that P2X3 receptors contribute to both the chemo-hyper reflexia and aberrant discharge of

93 carotid body activity in hypertension and predict that their blockade would cause an
94 anti-hypertensive effect. To address this, we have used highly selective P2X3 receptor
95 antagonists. Herein, we demonstrate the translatability of our pre-clinical findings to
96 hypertensive humans.
97

Results

Hyperactivity of carotid bodies in hypertensive rats

Carotid body afferent discharge was recorded from the carotid sinus nerve of the *in situ* preparation. Both basal discharge and chemoreflex evoked volleys (NaCN used to simulate hypoxia, 15—30 μg i.a.) were elevated in hypertensive versus normotensive rats (SH vs. Wistar: basal, 24.9 ± 2 vs 1.4 ± 0.3 spikes/s, respectively, $P < 0.001$; chemoreflex, 62.3 ± 3 vs 28.3 ± 2 spikes/s, respectively; $P < 0.001$, **Fig. 1a—c**). Next, low dose dopamine was used to inhibit carotid body discharge[39]. In SH rats *in vivo*, dopamine infusion (10 $\mu g/kg/min$ i.v. as per[39]) depressed respiration, an effect not observed in Wistar rats (**Fig. 1d**), indicating the presence of carotid body tonicity in the hypertensive animals. Intracellular recordings revealed that physiologically characterised chemoreceptive primary afferent petrosal ganglion neurones from hypertensive rats *in situ* were more depolarised (-51.5 ± 0.9 vs -55.7 ± 1 mV; $P < 0.001$, **Fig. 2a,c** and **Supplementary Fig. 1a**), exhibited tonic firing (1.5 ± 0.8 vs 0 Hz; **Fig. 2a**) and displayed an enhanced chemoreflex evoked firing response to an equivalent dose of sodium cyanide (22.5 μg) compared to normotensive rats (123 ± 5 vs. 52 ± 3 spikes; $P < 0.001$, **Fig. 2a,d**). In sum, compared to the normotensive rat, the carotid body of SH rats is hyperactive generating both aberrant tone and hyperreflexia.

Chemoreceptive petrosal neurone excitability in SH rats

We next compared the electrical excitability of chemoreceptive petrosal neurones and found that SH rats exhibited enhanced excitability to injected current pulses; for example, a 1nA current pulse evoked 25 ± 1.1 spikes vs 15 ± 1.7 spikes in Wistar rats (**Supplementary Fig. 2a,b**, $n = 6$; $P < 0.05$). Neither the membrane input resistance of

these cells (119 ± 3 vs 117 ± 9 MΩ; **Supplementary Fig. 1c**) nor whole cell capacitance was different between rat strains (**Supplementary Fig. 1d**).

Normalizing petrosal neurone excitability in SH rats

As P2X3 receptors are found in the carotid body of normotensive rats[36] and associated with pathological afferent sensitisation in numerous other organs[28,29,33] we assessed whether they were involved in carotid body dysfunction in SH rats *in situ*. In SH rats, focal delivery via a glass micropipette inserted into the ipsilateral carotid body of a highly selective non-competitive P2X3 receptor antagonist (AF-353; 15 *nl*, 20 μM[40]) caused hyperpolarisation (-9.9 ± 2.1 mV; n = 12, P < 0.001, **Fig. 2b,c**), total abolition of ongoing activity (**Fig. 2b**) and reduced chemoreflex sensitivity of petrosal chemoreceptive neurones (from 123 ± 5 to 34 ± 2 spikes; n = 12, P < 0.001, **Fig. 2b,d**). In normotensive animals *in situ*, AF-353 also caused hyperpolarisation (-4.4 ± 1.8 mV; n = 10, P < 0.001, **Fig. 2c**) and reduced the chemoreflex evoked volley from 52 ± 3 to 32 ± 2 spikes; n = 10, P < 0.001, **Fig. 2b,d**). Both the magnitude of the hyperpolarisation and reduction in chemoreflex evoked spiking were greater in SH versus Wistar rats (P < 0.001, **Fig. 2c,d**). Notably, after P2X3 receptor antagonism, carotid body activation yielded comparable firing responses in petrosal neurones between rat strains (SH: 34 vs. Wistar 32 spikes; NS), which was reversible in SH rats (**Fig. 2b**). After AF-353 treatment the intrinsic firing response to injected depolarising current pulses was reduced in SH (e.g. at 1 nA: 25 ± 1 to 19 ± 1 spikes; P < 0.05, **Supplementary Fig. 2a,b**) but not Wistar rat neurones (15 ± 2 to 12 ± 2, NS). We next tested whether P2X3 receptors were upregulated in SH rat petrosal neurones.

Upregulated P2X3 receptors in petrosal neurones of SH rats

Single cell PCR analysis of characterised chemoreceptive petrosal ganglion neurones recorded from SH rats *in situ* revealed a >4 fold up regulation of *P2x3* receptor mRNA relative to Wistar rats (**Fig. 2e,f**; $n = 6$, $P < 0.001$); this was confirmed at the protein level using western blot performed on carotid bodies (**Fig. 3a**). Interestingly, no change was found in *P2x3* (or *P2x2*) receptor mRNA expression in non-chemoreceptive petrosal neurones (**Supplementary Fig. 2e**) indicative of specificity to chemoreflex neurones. There was no rat strain related change in: *P2x2* receptor (**Fig. 2f**) and tyrosine hydroxylase (a known marker of glomus cells) mRNA expression in petrosal chemoreceptive neurones of SH rats (**Supplementary Fig. 2c**) or in chemoreceptive neurones located in the nucleus tractus solitarii (**Supplementary Fig .2f**). **Fig. 3b** shows co-localisation of P2X3 receptors with tyrosine hydroxylase immunopositive glomus cells (and axons, **Supplementary Fig. 3a,b**). Based on the up regulation of *P2x3* receptors in the petrosal neurones of SH rats, we tested whether there was a difference in sensitivity of these neurones to ATP between the rat strains.

Sensitisation of SH rat chemoreceptive petrosal cells to ATP

Application (15 *nl*) of ATP or α–β–methylene-ATP focally to the carotid body produced dose-dependent increases in the firing and membrane depolarisation of petrosal neurones. Notably, the petrosal neurones in the SH rat were more sensitive than those from Wistar rats: for ATP the $EC_{50\%}$ SH vs Wistar rat was 8.8 ± 2.2 vs 17.2 ± 1.3 ($P < 0.001$; **Fig. 3c,d**) and for α–β–methylene-ATP the $EC_{50\%}$ SH vs Wistar rat was 9.4 ± 2.1 vs 16.2 ± 2.4 ($P < 0.05$; **Supplementary Fig. 4a,b**). These findings were consistent with greater P2X3 receptor-sensitive inward current evoked by ATP in chemoreceptive petrosal neurones (**Fig. 3e,f**). Next, we tested if antagonism of carotid body P2X3 receptors lowered arterial blood pressure in SH rats.

Blocking P2X3 receptors systemically is anti-hypertensive

One hour infusions of a highly selective non-competitive P2X3 receptor antagonist,AF-219, were made at 1, 4 and 8 mg/kg/h, i.v.. This compound was preferred to the structurally related, more lipophilic antagonist AF-353, as AF-219 has been used clinically for other indications in humans[29], and since it does not cross the blood brain barrier significantly its use *in vivo* was preferable. We performed a PK analysis to assess plasma concentrations in SH rats (**Fig. 4a** and **Supplementary Fig. 5**) and subsequently correlated these with the falls in systolic blood pressure (**Fig. 4a**). In SH rats, basal arterial pressure was 151±3/109±3 (SBP/DBP) mmHg. AF-219 produced dose-dependent falls in arterial pressure (**Fig. 4a**); no response was observed in Wistar rats (**Fig. 4b**; **Supplementary Fig. 6**). At a dose of 8mg/kg/h SBP/DBP in SH rats fell by -28±3/-26±4 mmHg (**Fig. 4a**; $n = 7$, $P < 0.001$). Heart rate and respiratory frequency also decreased (by -38±8 and -12±3 /min, respectively; **Supplementary Fig. 6,** $n = 7$, $P < 0.01$). Interestingly, after carotid body resection a residual fall of -17±3 mmHg in SBP persisted with AF-219 infusion in SH rats (30 min, 8mg/kg/h; **Fig. 4b**, $n = 7$, $P < 0.05$; **Supplementary Fig. 7**). The chemoreflex evoked increase in arterial pressure in conscious SH rats was also reduced after systemic blockade of P2X3 receptors by AF-219 (bolus doses given i.v. >1 mg/kg; $n = 4$, $P < 0.05$; **Fig. 4c**). We next determined the mechanisms by which arterial pressure was lowered in SH rats by P2X3 receptor blockade.

P2X3 receptor blocker reduces sympathetic tone in SH rats

We evaluated the effect of P2X3 receptor antagonism on cardiovascular autonomic activity. Ongoing basal levels of thoracic SNA recorded *in situ* were reduced from

20.8±2.2 to 13.7±2.5 μV (**Fig. 5a—c**; n = 12, P < 0.001) following focal delivery of AF-353 (15 *nl*, 20 μM) to carotid bodies bilaterally in SH rats. This reduced value was similar to levels in normotensive rats either before (12.3±2.6 μV) or after AF-353 application (11.7±2.3 μV, **Fig. 5c**; n = 12, NS). Chemoreflex evoked increases in expiratory modulated SNA were 209±12% and 131±11% for SH and Wistar rats respectively, and after AF-353 were reduced significantly to 76±11% and 69±7.8% in SH and Wistar rats, respectively (**Fig. 5a—e**, n = 12, P < 0.001); these new basal values were not different to one another. Following drug washout, the depressant effects on SNA was reversed (basal and reflex evoked; **Fig. 5b**).

We replicated these data using TNP-ATP (**Supplementary Fig. 8**). a high affinity, non-selective P2X receptor antagonist that inhibits P2X1, P2X3 and heteromeric P2X2/3 with a 1000-fold selectivity over P2X2, P2X4 and P2X7 receptors that is structurally distinct to AF-353. This evidence supports AF-353 blocking P2X3 homomeric and P2X2/3 heterotrimeric receptors rather than non-selective actions on ionic conductances and/or other receptors.

Recordings were made from renal sympathetic nerves using radio-telemetry in conscious SH rats *in vivo* during chronic systemic P2X3 receptor antagonism using AF-219. This reduced both the basal renal sympathetic activity (-37±13 normalised units relative to baseline, **Fig. 5f;** P < 0.05) and the chemoreflex evoked increase in renal sympathetic activity (**Fig .5g,h;** P < 0.05). Spontaneous renal sympathetic baroreflex gain was enhanced after AF-219 (0.14±0.07 *vs* 0.01±0.02 %.mmHg^{-1}; P < 0.05) but not vehicle infusion. Although no changes in autonomic activity to the heart were detected in conscious SH rats after AF-219 infusion (**Supplementary Fig. 9a,b**), spontaneous

cardiac baroreflex was increased (0.13±0.04 to 0.27±0.05 ms/mmHg, **Supplementary Fig. 9c;** $P < 0.001$). We next tested the translatability of the SH rat data and potential use of P2X3 receptor antagonist in human hypertension.

Translating carotid body P2X3 receptors to human hypertension

If P2X3 receptors within the carotid body are to be considered a novel anti-hypertensive target in humans, then evidence for their presence and pharmacokinetics of suitable antagonist are essential first steps. Carotid bodies were obtained from cadavers and processed for P2X3 receptors immunocytochemistry ($n = 5$) and western blot ($n = 4$). P2X3 receptor antibody specificity was determined (**Supplementary Fig. 10**). P2X3 receptor expression was prevalent in human carotid body (**Fig. 6a** and **Supplementary Fig. 11a**) showing co-expression with tyrosine hydroxylase, a marker for glomus cells. Axons within the carotid body also expressed P2X3 receptors (**Supplementary Fig. 11b**). This expression pattern was similar to that seen in the SH rat (**Fig. 3b**).

With the presence of P2X3 receptors in hypertensive human carotid body, we next assessed whether there was tonicity in the carotid body of hypertensive humans by chemical and reversible inactivation of it. In six hypertensive humans, minute ventilation was measured during low dose dopamine infusion (5 min; 2 *μg/kg/min* i.v.) as described previously to inactivate the carotid body[38]. A bi-phasic response in minute ventilation to dopamine was observed (**Fig. 6c**) comprising a depression (-1.28± 0.52 L/min; $P < 0.05$) and over shoot following dopamine (1.28± 0.41 L/min; $P < 0.05$) relative to vehicle infusion. These data indicate that the carotid body of hypertensive humans can generate tonic drive and that dopamine infusion allows identification of it.

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Discussion

Many transmitter mechanisms mediating reflex responses to hypoxia have been described within the carotid body and may contribute to its sensitisation in disease states; these include: TASK and ASIC channels[14,25], hydrogen sulphide[23,24], carbon monoxide[24], nitric oxide[24] and carotid body blood flow[27]. However, *in vitro* studies have also shown a major role for ATP release from glomus cells in response to hypoxia in both rat[30], cat[31] and human[35]. Whether there is a link between ATP release and any of the aforementioned mechanisms remains an open question. Moreover, P2X2 and P2X3 receptor subunits were both found to be present in the rat carotid body[36] and using co-cultures of glomus and petrosal cells *in vitro* Nurse and colleagues demonstrated that ATP acted via P2X receptors to excite petrosal neurones[36]. This evidence was based on using suramin, a weak and non-selective P2X antagonist. P2X2-containing channels (P2X2 homotrimers and/or P2X2/3 heterotrimers) likely dominate the normal physiological ventilatory response to hypoxaemia as the P2X2 single and P2X2 + P2X3 double KO mice, but not the P2X3 single KO, showed ventilatory hyporeflexia[37]. Thus, P2X3 subunit expression changes seem associated with pathological sensitisation of carotid body reflexes.

In the present study, using selective antagonists, we found that both exogenous and endogenous ATP released from the carotid body can act on P2X3 subunit containing receptors on petrosal neurons. A caveat is that we cannot fully establish the relative contribution of homotrimeric P2X3 versus heterotrimeric P2X2/3 receptors in such responses, although the antagonists we used have higher affinity for P2X3 homotrimeric receptors[28], and a P2X3 heterotrimeric upregulation in carotid body could account for increased ATP sensitivity per se, as this channel form in recombinant studies displays

273 significantly lower EC_{50} values to the nucleotide[40]. Our voltage clamp data indicate a
274 non-desensitising current to α-β-methyl ATP and probable involvement of P2X3
275 homomeric and P2X2/3 heteromeric receptors. Our data provide the first evidence that
276 P2X3 receptors in the carotid body play a vital role in controlling its reflex sensitivity in
277 SH but not normotensive rats.
278
279 Up-regulation of P2X3 receptors in the carotid body appears, in part, causal for the
280 aetiology of the tonicity, hyperreflexia of chemoreceptive petrosal neurones and
281 downstream hyperactivity of the sympathetic nervous system in SH rats. We
282 acknowledge that chemoreceptive petrosal neurones may have a larger receptive field
283 in the carotid body of SH vs Wistar rats contributing to their hyperactive state. Notably,
284 upregulated *P2x3* receptor mRNA was exclusive to chemoreceptive petrosal neurones
285 (i.e. not upregulated on non-chemoreceptive petrosal cells). The carotid body tonicity
286 we report from *in situ* preparations (1.5 Hz per petrosal neuron) most likely
287 underestimates that in conscious SH rats as recordings were made in hyperoxia, a
288 condition known to temper carotid body discharge[14,20]; this may also explain the dearth
289 of activity in neurones recorded from Wistar rats. Whether P2X3 receptor up-
290 regulation alone is sufficient for the pathological carotid body signalling in the SH rat
291 (without the need for increased ATP release or reduced ATP clearance) is unclear.
292 However, given that the microvasculature of the carotid body is hypertrophied in the SH
293 rat[41] and the possibility that arterioles feeding the carotid body receive high
294 sympathetic drive[41], the tissue is likely to be hypoxic/hypercapnic – a condition known
295 to induce ATP release[42]. Additionally, inflammation, known to be present in the carotid
296 body of SH rats[41], will also drive ATP release[43].
297

The association of P2X3 receptor up-regulation and aberrant afferent signalling is not new[28] but consistent with sensitisation of visceral sensing mechanisms from, for example, the bladder[44,45], urinary tract[46], larynx [29], lungs[47], gastro-intestinal-tract[48,49] and skeletal muscle[50] in numerous diseased states including chronic pain[51]. Elucidation of the mechanisms controlling *P2x3* receptor expression at both transcriptional and cytosolic trafficking levels within the carotid body and other organs now becomes an important issue to allow translation into the clinical arena.

The finding that P2X3 receptor activation evoked a rapidly inactivating current *in vitro*[52] seems inconsistent with an ability of P2X3 receptors to maintain tonic drive from the carotid body (or any organ). Notably, low pH, a postulated condition in the carotid body of SH rats (see above;[41]), can potentiate P2X3 receptor inward currents and reduce de-sensitisation[53]. Furthermore, the concept has been developed that P2X3 receptor activation may lead to local generator potentials and calcium transients in afferent terminals that lowers the threshold for activation by any other excitatory agent, as described in somatosensory nociceptor C-fibres as leading to a condition of hyperalgesic priming[54,55]. Such threshold attenuation and chemoceptive priming may be under the control of P2X3 receptor in the carotid body, although we do not rule out possible emergence of P2X2/3 heteromeric receptors, as these channels show less rapid de-sensitisation[52], which is consistent with our voltage clamp data (**Fig. 3e,f**).

We found a robust dose-dependent lowering of arterial pressure following systemic administration of AF-219 in SH rats. That a fall in blood pressure was seen in rats with Goldblatt hypertension (W. Pijacka, F.M. McBryde & J.F.R. Paton – unpublished) suggests this is not restricted to the SH rat. We propose that the effect of P2X3 receptor

antagonism is, in most part, acting within the carotid body but not exclusively as the antagonist induced fall in arterial pressure was substantially reduced but not abolished after carotid body ablation (**Fig. 4b** and **Supplementary Fig. 7**). Further support for a direct action of the antagonist at the carotid body is that the fall in arterial pressure was accompanied with a reduction in sympathetic activity *in vivo*, a mechanism for the arterial pressure reduction, which was also found following direct injection of AF-353 into the carotid body *in situ*. The remaining residual depressor response seen with AF-219 after carotid body ablation suggests a contribution from other sensory afferents in which P2X3 receptors have become active. We propose this includes glomus tissue in the thorax and abdomen but this remains to be confirmed. Mechanistically, the decrease in sympathetic activity included a reduction in its respiratory modulation, particularly its expiratory component. It is this component that is upregulated in numerous models of hypertension[56,57] supporting the importance of the central respiratory generator as a major driver of autonomic imbalance in hypertension[57]. We also found an improvement in the spontaneous cardiac baroreceptor reflex, possibly reflecting the removal of the inhibitory influence of the carotid body on the carotid sinus baroreceptor reflex as reported previously in both SH rats[20] and human hypertensives[58]; this may contribute to the sympatho-inhibition and anti-hypertensive response of P2X3 receptor antagonism in the SH rat.

The clinical significance of the synergy of up-regulated P2X3 receptor expression in the carotid body and petrosal neurones with emerging pathology should not be underestimated. It follows that administration of a P2X3 receptor antagonist should attenuate aberrant signalling without affecting physiological function. Data herein supports this logic, as carotid body tonic drive and hyperreflexia were abated yet the

system remained physiologically responsive to stimulation akin to control animals (**Fig. 2b**). This is entirely consistent with a recent human trial to treat chronic pathological cough where the latter was abated but physiological/protective cough reflexes did not appear to be altered[29]. Peripheral chemoreception also has both regulatory and protective reflex functions[59]. Simultaneously abolishing pathological signalling while preserving normal carotid body function with a P2X3 antagonist such as AF-219 is an ideal outcome clinically that would be paramount in drug safety and target specificity. An issue may be the known adverse effect of P2X3 receptor antagonism affecting taste perception[29] but appropriate dosing may negate this.

Over the last 20 years there has been a dearth of novel anti-hypertensive therapeutics (e.g.[7]). We speculate that P2X3 receptor antagonism may be most efficacious in a sub-population of uncontrolled hypertensive humans with demonstrable aberrant carotid body activity and an overactive sympathetic nervous system. The existence of P2X3 receptors in the carotid body of hypertensive human, the release of ATP from human glomus cells in response to hypoxia[35] and the ability of dopamine to select those individuals with aberrant carotid body discharge (**Fig. 6c**) supports our contention of a translational trial to test the anti-hypertensive effect of P2X3 antagonism; this study now awaits commencement.

Methods

Methods and any associated references are available in the online version of the paper.

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Author contributions

W.P. conducted all the *in vivo* radio-telemetry blood pressure studies, the rat and human immunocytochemistry and western blotting; this also included data analysis, figure and manuscript preparation. D.J.A.M. performed all *in situ* rat nerve and petrosal neurone whole cell recording studies; this also included data analysis and figure preparation. M.P.d.S. performed the single neurone PCR study. L.E.K.R. with A.K.N. carried out the diagnosis and recruitment of humans with hypertension and, L.E.K.R. with E.C.H., performed and analysed data from the dopamine infusion study. B.H.M. supported all *in situ* studies, assisted in experimental design, data analysis and manuscript preparation. F.D.M. conducted the *in vivo* radio-telemetry study for recording renal sympathetic nerve activity; this also included data analysis and figure preparation. A.P.A. performed

some of the first immunohistochemistry on human carotid bodies. A.P.F. provided the P2X3 receptor antagonists, carried out the PK analysis, assisted in drug trial design and manuscript preparation and revision. J.F.R.P. orchestrated the design of the project, provided supervision with data acquisition and analysis, wrote the manuscript and revised it.

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Competing financial interests

A.P.F. is Chief Scientific Officer for Afferent Pharmaceuticals. The other authors declare no competing financial interests.

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618 **Figure legends**

619

620 Figure 1

621 Overactive peripheral chemoreceptors in spontaneously hypertensive (SH) rats.
622 Original recordings of carotid sinus nerve (CSN; (**a**)) activity showing both basal
623 discharge (**a**,**b**) and that evoked reflexly by stimulating the carotid body with sodium
624 cyanide (**a**,**c**; 22.5 µg NaCN i.a., arrowed in **a**) in SH and Wistar rats. CSN data from the *in*
625 *situ* perfused preparation. (one-way ANOVA Bonferroni post-test; n = 10 or 11, ***P <
626 0.001). (**d**), dopamine infusion (10 $\mu g/kg/min$ i.v.) while recording ventilation
627 frequency in conscious radio-telemetered Wistar and SH rats (n = 5 each) before and
628 after selective carotid body resection (SHR CBR). One-way ANOVA Dunnett's post-test.
629 * P < 0.05. ***P < 0.001. All data are mean ± s.e.m.

630

631 Figure 2

632 P2X3 receptor mediated hyperreflexia and tonicity of chemoreceptive petrosal
633 neurones in spontaneously hypertensive (SH) rats are associated with upregulation of
634 *P2x3* receptor mRNA. (**a**), two representative whole cell patch clamp recordings from
635 chemoreceptive petrosal neurones from a Wistar (left) and SH rat (right) recorded in
636 the *in situ* preparation. Ongoing discharge, membrane potential and reflex evoked
637 responses to carotid body stimulation (NaCN, sodium cyanide, 22.5 μg i.a. arrowed)
638 were compared between rat strains. (**b**), responses of neurones in (**a**) following P2X3
639 receptor blockade with AF-353 (20 μM, 20 nl), which was delivered focally into the
640 carotid body by picoinjection. Effects in SH rats were reversed upon washout. Grouped
641 mean data summarising rat strain related differences and responses in membrane
642 potential (**c**; see also **Supplementary Fig. 1a,b**) and chemoreflex evoked firing

responses (**d**) before and after P2X3 receptor antagonism. Data in (**c**) are mean ± s.d. and those in (**d**) are mean ± s.e.m. One-way ANOVA Bonferroni post-test (n = 12 SH, n = 10 Wistar rats). The difference in expression of *P2x3* and *P2x2* receptor mRNA from petrosal chemoreceptive neurones (identified using sodium cyanide, NaCN, 22.5 μg, i.a.) during whole cell patch (**e**) was revealed using single cell PCR ((**f**); n = 5 or 6). Two-way ANOVA Bonferroni post-test. Data in (**f**) are mean ± s.e.m and were generated from the *in situ* arterially perfused preparation. *** $P < 0.001$.

Figure 3

In spontaneously hypertensive (SH) rats P2X3 receptor protein was upregulated in carotid body and chemoreceptive petrosal neurones were sensitised to ATP relative to Wistar rats. (**a**), difference in P2X3 receptor protein in the carotid body of Wistar relative to SH rats (Mann–Whitney t-test; n = 4 or 5). (**b**) P2X3 receptor immunofluorescence labelling on glomus cells identified by the presence of tyrosine hydroxylase (TH; n = 3 Wistar, n = 3 SH rats). See, **Supplementary Fig. 10** for control data on the specificity of the P2X3 receptor antibody. Scale bars 100 μm (top left) and 25 μm all others. (**c**), ATP microinfused into the carotid body while whole cell recording (current clamp) from petrosal chemoreceptive neurones in Wistar (n = 10) and SH rats (n = 13). (**d**), rat strain comparison of the ATP (applied to the carotid body) evoked membrane depolarisations from petrosal chemoreceptive neurones in both rat strains. AUC, area under the curve. (**e**), voltage clamp data indicating the ATP (applied to the carotid body) evoked inward current from identified petrosal chemoreceptive neurones before and after application to the carotid body of the P2X3 receptor antagonist (AF-253, 20 μM) and the non-selective P2X receptor antagonist, suramin (100 μM) in Wistar (top traces*;* n = 12) and SH rats (bottom traces*;* n = 12). (**f**), rat strain differences in the

AF-353 sensitive ATP evoked inward current. Neurophysiological data were from the *in situ* arterially perfused preparation and comparisons made using an unpaired t-test. All data are mean ± s.e.m. * $P < 0.05$; *** $P < 0.001$.

Figure 4

P2X3 receptor antagonism lowers arterial pressure in conscious spontaneously hypertensive (SH) rats. (**a**), Intra-venous infusions of a P2X3 receptor antagonist (AF-219, 1—8 mg/kg/h) showing dose-dependent responses in systolic blood pressure (SBP). Solid lines indicate the blood pressure responses and dotted lines the predicted PK value based on plasma sampling (see also **Supplementary Fig. 5**). (**b**), Comparison of the SBP responses induced with 8 mg/kg/h AF-219 i.v. in SH ($n = 7$) and Wistar rats ($n = 7$), and in SH rats before and after carotid body resection (CBR; $n = 7$). (**c**), the peripheral chemoreflex evoked pressor responses in SBP during infusion of vehicle and 0.5—8 mg/kg/h AF-219. Data from seven conscious radio-telemetered rats are mean ± s.e.m. One-way ANOVA Dunnett's post-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5

The anti-hypertensive action of P2X3 receptor antagonism is associated with a reduction in sympathetic activity in spontaneously hypertensive (SH) rats *in situ* and *in vivo*. A comparison of the effect of a P2X3 receptor antagonist (AF-353; 20 μM, 15 *nl*) applied locally to both carotid bodies on the ongoing thoracic chain sympathetic activity (arrow, raw: tSN; integrated: ∫tSN) and the sympathetic nerve reflex response (arrow head) to peripheral chemoreceptor stimulation (evoked by NaCN, 22.5 μg i.a.) in 12 Wistar (**a**) and 12 SH rats (**b**). The effect of P2X3 receptor antagonism within both carotid bodies on the basal (**c**), inspiratory- (**d**) and expiratory (**e**) modulated tSN for

693 both rat strains is shown (n = 12 each). Inspiratory and expiratory modulation of tSN
694 was determined using averaged triggering from the phrenic nerve (PN). One-way
695 ANOVA Bonferroni post-test; data are mean ± s.e.m. from *in situ* rat preparations (n = 12
696 each strain). (**f**), renal sympathetic nerve activity (RNA) recorded from conscious radio-
697 telemetered *in vivo* SH rats (n = 5) during infusions of AF-219 (8 mg/kg/h i.v.). Note the
698 similar time course of the response in RNA to the fall in systolic blood pressure depicted
699 in **Fig. 4(a)**. A representative example of the peripheral chemoreflex evoked
700 sympathoexcitatory response (NaCN 22.5 μg i.a.) in a conscious radio-telemetered SH
701 rat before and after AF-219 (**g**) with mean data shown in (**h**), which includes drug
702 washout data. Repeated measures one-way ANOVA, with Holm-Sidek post-hoc
703 comparison. n = 5, * $P < 0.05$, *** $P < 0.001$.
704
705
706 Figure 6
707 P2X3 receptor expression and aberrant tone generation in hypertensive human carotid
708 bodies. (**a**), on the left is a section of a carotid body from a human cadaver showing
709 P2X3 receptor immunofluorescence (green) with the nuclear stain - DAPI (blue; scale
710 bar 100 μm). On the right, high power confocal images showing co-localisation of P2X3
711 receptors, tyrosine hydroxylase (TH) and DAPI (scale bar 25 μm). Data repeated in five
712 human carotid bodies. See **Supplementary Fig. 10** for P2X3 receptor antibody control
713 and **Supplementary Fig. 11** for axonal labelling. (**b**), western blot of the P2X3 receptor
714 protein in human carotid body (n = 4). (**c**), low dose dopamine infusion (2 μg/kg/h i.v.)
715 was used to inactivate the carotid bodies in six awake hypertensive humans (each
716 colour coded) while recording minute ventilation in the supine position. A dextrose
717 vehicle infusion was used as control and reference at time zero. The mean (± s.e.m.)

response is indicated by the black dotted line. Note appearance of a rebound hyperventilatory response post-dopamine infusion. One-way ANOVA Bonferroni post-test. * $P < 0.05$ vehicle versus peak depression; ** $P < 0.01$, depression versus rebound (mean of 5 min); † $P < 0.05$, vehicle versus rebound.

Online Methods

A. Animal studies

All procedures conformed to the UK Animals (Scientific Procedures) Act 1986, and were approved by the University of Bristol ethical review committee. We used male spontaneously hypertensive (SH) or Wistar rats, which were bred within the animal facility of the University of Bristol. Animals were selected from different litters and housed with controlled temperature (21 ± 2 ºC) and humidity (55 ± 10%). Wistar and SH rats were exposed to 12 hour day-night shift with unlimited access to food and water. We used rats either 4-5 weeks old (70-100 g) for *in situ* preparations or 14-16 weeks for *in vivo* radio-telemetry. Power calculations were based on: determining response magnitude (arbitrarily or based on previous reports or pilot experiments), data variance (our previous experience, published papers, pilot experiments), taking into consideration number of drugs to be tested and drug doses, and expected technical success rate of each type of experiment. Given the highly technical nature and longevity of the studies it was not possible to blind them.

Arterially perfused in situ juvenile rat preparation

Male Wistar or SH rats, 4-5 weeks, weighing 70–100 g were prepared as originally described[60]. In brief, rats were anesthetized deeply using isoflurane (5%). Anesthetic depth was assessed by a failure to respond to a noxious pinch of either a paw or the tail. Anesthetized rats were transected below the diaphragm, their upper body submerged in ice-cooled Ringer solution and decerebrated pre-collicularly by gentle aspiration. The preparation was skinned, transferred to a recording chamber and a double-lumen catheter was inserted into the descending aorta. One lumen was used to deliver

perfusate pumped using a roller pump (Watson Marlow 505S). The perfusate was an isosmotic Ringer solution (containing in mM: NaCl 120, $NaHCO_3$ 24, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1.25, KH_2PO_4 1.25, glucose 10) containing an oncotic agent (polyethylene glycol, 1.5%; Sigma-UK), gassed with carbogen (95% O_2 and 5% CO_2), warmed to 32 °C, pH 7.3 after carbogenation, and filtered using a nylon screen (pore size: 25 μm diameter). A side connector to this catheter allowed administration of drugs directly into the arterial circulation. The second lumen of the catheter was used to monitor aortic perfusion pressure. The left phrenic nerve was isolated and its activity (PNA) recorded from the cut central end using a glass suction bipolar electrode held in a 3-D micromanipulator. Rhythmic ramping PNA gave a continuous physiological index of preparation viability. After respiratory-related movements commenced, a neuromuscular blocker (vecuronium bromide, 40 $\mu g.ml^{-1}$, Norcuron Organon Teknika) was added to the perfusate to stabilize mechanically the preparation. Sympathetic nerve activity (SNA) was also recorded from the thoracic or lumbar sympathetic chain using a bipolar glass suction electrode. All nerve signals were AC-amplified, band-pass filtered (0.5 Hz−5 kHz), rectified and integrated and sampled at 2-5 kHz. At the end of each experiment the noise level was measured after application of lidocaine (2%) to the sympathetic chain; this level was subtracted from the integrated signal. The head of the preparation was fixed by ear bars.

Whole cell recordings from chemoreceptive neurones

The carotid body/carotid sinus nerve/petrosal ganglion complex was isolated on the animal's right side. Using recently described techniques[57,61] whole cell patch clamp recordings of chemoreceptive petrosal ganglionic or NTS neurones was performed with electrodes filled with a solution containing the following (in mM): 130 K-gluconate, 4.5

775 $MgCl_2$; 14 trisphosphocreatine, 10 HEPES; 5 EGTA; 4 Na-ATP; 0.3 Na-GTP; pH 7.3, and
776 ~300 mOsmol and had resistances of 3–8 MΩ when tested in bath solution. Current-
777 and voltage- clamp experiments were performed using an Axopatch-200B integrating
778 amplifier (Molecular Devices) and pClamp acquisition software (version 10.0, Molecular
779 Devices). Gigaseals (>1 GΩ) were formed, and whole-cell configuration was obtained by
780 suction. To allow stable whole cell recordings, the petrosal ganglion was opened along
781 its lateral aspect. A mesh grid was lowered onto the ganglion for stabilization, while
782 permitting visualisation of the petrosal ganglia. We used electrical stimulation of the
783 carotid sinus nerve (the axons of petrosal neurones) to find the chemosensitive petrosal
784 and NTS neurones as characterised by their excitatory response to sodium cyanide
785 injected into the aorta (0.03%, 50 μl). ATP (15 *nl* of 0.5, 2, 10, 20, 40, 100 mM) or α-
786 β–methylene ATP (15 *nl* of 0.5, 2, 10, 20, 40, 100 mM) or AF-353 (15 *nl* of 20 μM; a P2X3
787 receptor antagonist)[40] were injected into the carotid body using a 2 μm tip diameter
788 glass microelectrode attached to a picopump (Picospritzer II, Parker Instrumentation).
789
790 ***Single petrosal neurone PCR***
791 The cytoplasm of the chemosensitive petrosal and NTS neurones were pulled into a
792 patch pipette with a slight negative pressure as described recently[61]. The cytoplasm was
793 then placed into a microtube containing High Capacity cDNA Reverse Transcription Kit
794 reagents (Life Technologies) and nuclease-free water for subsequent transcription in a
795 thermocycler (Mastercycler Gradient, Eppendorf). A pre-amplification of the cDNA was
796 performed using the TaqMan PreAmp Master Mix Kit (Life Technologies) with the
797 following probes: Rn04219592_g1 (*P2x2*), Rn00579301_m1 (*P2x3*),
798 TH: Rn00562500_m1, Post-synaptic density-95: Rn00571479_m1 and β-Actin:
799 NM_031144.2 (reference gene). The pre-amplification protocol consisted of a hold

temperature at 95°C during 10 minutes and 14 cycles of 95°C and 60°C during 15 s and 14 minutes, respectively. The reactions for the single-cell qRT-PCR were performed in singleplex and triplicate (StepOnePlus System, Applied Biosystems) using the same probes described above and the TaqMan Universal PCR Master Mix kit (Life Technologies) according to the manufacture's recommendations. Water was used instead of cDNA as a negative control. β-actin was used as a control gene to normalise the reactions. The relative quantitation was determined by the $\Delta\Delta C_t$ method. For each sample, the threshold cycle (C_t) was determined and normalised to the average of the housekeeping genes ($\Delta C_t = C_{t_{\text{Unknown}}} - C_{t_{\text{referencegene}}}$). The fold change of mRNA content in the sample of petrosal neurones from SHR relative to the Wistar group was determined by $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_{t_{\text{Unknown}}} - \Delta C_{t_{\text{Control}}}$. Data are presented as mRNA expression relative to the Wistar group. Dual probing of mRNA included: TH and PSD-95 in 11 petrosal chemoreceptive cells from Wistar rats; TH and PSD-95 in 9 petrosal chemoreceptive cells from SHR; *P2x2* and *P2x3* receptor expression in 6 petrosal chemoreceptive cells from Wistar and 5 petrosal chemoreceptive cells from SHR.

In vivo blood pressure and renal nerve monitoring

Most surgical techniques were described previously[20]. Rats were anaesthetised with ketamine (100mg/ml; Vetalar, Zoetis, London, UK)/medetomidine hydrochloride (1 mg/ml Domitor, Elanco Animal Health, Hampshire, UK) injected intra muscularly.

For blood pressure recording only, an incision was made in the midline of the abdomen and the abdominal aorta was exposed and cannulated just above the iliac bifurcation. The cannula of the transmitter (PA-C40; DSI, USA) was inserted until the tip rested just below the left renal artery branch point, and held in place using a tissue adhesive (VetBond, 3M, USA) and cellulose matrix. The transmitter body was placed in the

abdominal cavity. Animals were allowed a seven days recovery period. A non-steroidal anti-inflammatory analgesic, was administered for 3 to 5 days postoperatively (0.006 mg/100g of Metacam, Boehringer Ingelheim, Germany). Some rats were fitted with heparinised (TDMAC, Plolysciences Inc., Eppelheim, Germany) catheters placed into right jugular and left femoral vein for blood sampling and drug infusions, respectively. The catheter placed in the right jugular vein was perforated according to the protocol[62]. Animals were allowed five days recovery post vein catheterisation. Catheters were flushed with heparinised saline solution (0.9 % Saline/ 100U Heparin) every other day in order to maintain their patency.

For renal nerve recording, rats were implanted with telemetric devices (TRM56SP; Millar Inc, Houston, Texas, USA). Details for blood pressure procedures are given above. For renal nerve recording, the right renal artery was exposed via a retroperitoneal approach, the renal nerves gently freed from surrounding connective tissue and a small piece of parafilm placed underneath. The renal nerve(s) were then lifted over bipolar silver wire recording electrodes, isolated with a biocompatible silicone elastomer (Kiwk-Sil, WPI, Eu), and the nerve/electrode complex secured in place using tissue adhesive and cellulose mesh. The incision was closed, and rats recovered for at least seven days post-operatively, with analgesic treatment for 3 days (buprenorphine, 0.01 mg/kg per dose).

Experimental protocols for in vivo rat studies

These studies were repeated by two researchers working independently. Drug infusions (i.v.) were carried out in rats housed singularly. Animals were acclimatised to experimental environment for at least 2 hours. At this time blood pressure remained

stable. AF-219 was dissolved in 0.9% saline containing 10 mM HCl (vehicle). In some animals in which arterial blood pressure was measured, a PK analysis of AF-219 was performed by withdrawing blood from the jugular catheter at two time points: one hour from the start of infusion and one hour post infusion. Rats received an infusion of vehicle and 1, 4 and 8 mg/kg/h AF-219 for 60 minutes at 5 ml/kg/h into their femoral vein followed by a 60 minute washout period. Administration order of the drug doses and vehicle was randomized. These doses were selected by modelling pharmacokinetic data to obtain a pharmacokinetic profile in rats similar to that achievable in humans. The modelling was validated by quantifying AF-219 plasma concentrations after the 60 minute infusion period by using an HPLC-MS/MS assay and stable-labelled AF-219 as internal standard. The peripheral chemoreflex was tested with sodium cyanide (0.1 ml bolus i.v. 0.04% NaCN; BDH, Poole, UK, flushed with 0.9% saline (0.1 ml)). A bolus injection of sodium cyanide (0.04% in 0.1 ml) was given 45 minutes before, and 60 and 90 minutes after the beginning of the infusion to check for chemoreflex sensitivity. Rats were allowed 24 hour recovery between vehicle/drug and each drug dose. Infusions were carried using a standard syringe pump (Harvard apparatus, MA, USA). Dopamine hydrochloride was infused (10 μg/kg/min; Sigma-Aldrich Company Ltd., Dorset, UK) to reversibly inactivate carotid bodies and assess their tonicity by measuring arterial pressure, heart rate and respiratory frequency.

Rats tested with NaCN and dopamine hydrochloride underwent selective bilateral carotid body resection. Via a midline incision through the skin on the ventral surface of the neck, and using a binocular dissecting microscope the carotid bifurcation was identified. From a medial perspective the carotid body was visualised and ablated using fine watchmakers forceps. Effective carotid body ablation was confirmed by an absence

of a cardiovascular and respiratory response to NaCN (i.v.). Animals were allowed six days for recovery. Once confirmation of selective carotid body ablation rats were infused with vehicle, AF-219 (8 mg/kg/h i.v.) and dopamine hydrochloride (10 µg/kg/min i.v.) on separate days.

Western blotting and immunocytochemical studies

Rat left and right carotid bifurcations were surgically removed from deeply anaesthetised rats and immediately transferred into ice cold saline. Carotid bodies were dissected under a Motic K Series Stereo Microscope (Ted Pella, Inc.). Five SH and four Wistar rats were used for western blotting and six SH and six Wistar rats were used for immunohistochemistry. Specificity of the P2X3 receptor antibody was validated (**Supplementary Fig. 10**). Carotid artery bifurcations were removed and carotid bodies dissected from these and snap frozen in liquid nitrogen (left and right together) for western blotting or they were fixed in ice cold methanol/DMSO (4:1) for immunohistochemistry. The relative expression of protein corresponds to one pair of carotid bodies (left and right). Immunohistochemistry was carried out on the whole mount rat carotid body and anti-P2X3 receptor antibody (APR026AN0202, Alomone, Israel). Incubation with anti-P2X3 and TH antibody was carried out in 2% goat serum over night at 4°C, gently mixed. All other details are given below (Human Studies) and similar to those used in the human studies.

Statistical analysis

Digitally transmitted arterial pressure and renal SNA signals were collected continuously during the drug or vehicle infusion period. Arterial pressure was

899 measured during resting conditions. The renal SNA signal was amplified, filtered (50-
900 5000 Hz), full-wave rectified and integrated using a low pass filter with a 20 ms time
901 constant. Arterial pressure and renal SNA were sampled at 1k Hz using a 1401
902 acquisition system and purpose-written scripts in Spike2 software (Cambridge
903 Electronic Designs, Cambridge, UK). From the arterial pressure we derived pulse
904 pressure, heart rate and respiratory frequency. Power spectral analysis was performed
905 on heart rate using Spike 2 software (CED instruments, Cambridge, UK). The inter-burst
906 interval in renal SNA was taken as the baseline noise level, and removed from the signal.
907 The renal SNA signal was then scaled, with the 30 minutes immediately prior to infusion
908 taken as the 100% normalised level. We report the change in renal SNA with AF-219
909 relative to vehicle. The mean differences between baseline and treatment were analysed
910 by the GraphPad Prism 6 (GraphPad Software, Inc., USA). We analysed the spontaneous
911 cardiac and renal sympathetic baroreflex gain (sBRG). The sBRG for the arterial
912 pressure–renal SNA relationship was calculated for 10 minutes immediately before, and
913 the final 10 minutes of the 60 minute infusion period of AF-219 or vehicle. Diastolic
914 pressure was smoothed over 5 beats, and positive or negative pressure ramps of at least
915 5 consecutive beats were automatically identified using Spike2 scripts (CED
916 instruments, Cambridge, UK). All ramps where both heart rate and renal SNA opposed
917 the change in the corresponding DBP ramp were included for analysis. As described
918 earlier, renal SNA was normalised with the baseline (pre-infusion) level set at 100%. A
919 paired student t-test was used to compare the renal SNA sBRG between vehicle and AF-
920 219 infusions.
921
922 The type of statistical test performed is indicated in the figure legends. Data were
923 normally distributed and variance was comparable between groups statistically

924 compared. Unless stated in the figure legends, data were expressed as mean ± SEM are
925 presented and data were assumed significant when $P < 0.05$.
926
927 **Drugs**
928 Two highly potent and selective non-competitive P2X3 (homotrimeric) and P2X3/P2X2
929 (heterotrimeric) receptor antagonists were used in these studies: AF-353 and AF-219.
930 Although the antagonists we used have higher affinity for P2X3 homotrimeric
931 receptors[28,40,44] they cannot fully establish the relative contribution of homotrimeric
932 P2X3 versus heterotrimeric P2X2/3 receptors. AF-353 was used in all *in situ* studies and
933 was only available to us when these were performed; this drug does cross the blood
934 brain barrier. The *in vivo* experiments used AF-219 as this is known not to cross the
935 blood brain barrier. The chemical formula for AF-219 is:
936 (amino-(4-isopropyl-2-methoxyphenyl)sulfone)-5-yl-(2,4-diaminopyrimidine-
937 5yl)ether. The structure of AF-219 is contained in a published patent (see:
938 http://pdfaiw.uspto.gov/.aiw?PageNum=0&docid=20150057299&IDKey=DA73323D44
939 81&). Doses used were based on IC_{50} data[63,64]. For recombinant P2X3 homotrimers the
940 IC_{50} for AF-353[64] and AF-219 was ~8 nM and ~30 nM, respectively. A higher IC_{50} was
941 found for P2X2/3 heterotrimeric receptors – for example 100 to 250 nM for AF-219.
942 Note that no inhibitory effect on any non-P2X3 subunit containing receptors was
943 noted[63,64].
944
945
946 **B. Human studies**

All participants gave informed consent to participate in the studies, which were approved by the Central Bristol Research Ethics Committee (REC numbers: 14/SW/0054 and 12/SW/0277).

Carotid body tone in hypertensive humans

Small increases in circulating dopamine can decrease peripheral chemoreceptor afferent input into the brainstem[65]. Thus, low dose intravenous dopamine infusion (2 μg/kg/min) was used to test for any tonicity from the carotid bodies. Six hypertensive humans (age 48.8±3 yr; BMI 28.6±4, three female) with an office blood pressure of >140/90 mmHg (ambulatory blood pressure: >139/91 mmHg) and on a mean of 2.83 anti-hypertensive medicines were selected. Participants were given an intravenous infusion of dextrose (5%) for 5 minutes via peripheral venous access while breathing room air as a vehicle control. After the vehicle infusion, the line was purged and the intravenous infusion of dopamine (infusion concentration: 100 μg/ml of dopamine in 5% dextrose) was started for 5 minutes while breathing room air. Equivalent volumes of dextrose and dopamine were administered. Participants were blinded to the order of the infusions. This was following by a recovery period of 5 minutes while breathing room air allowing time for the dopamine to be metabolised. Respiratory volumes were measured via a respiratory flow-head connected to the expiratory port of a non-rebreathing valve attached to a face mask. Data were acquired using PowerLab and LabChart 7 software (AD Instruments) and analysed to calculate respiratory rate, tidal volume and minute ventilation using Spike2 (Cambridge Electronic Design) and MATLAB (MathWorks). The data was analysed by GraphPad Prism 5 (GraphPad Software, Inc., USA).

Western blotting and immunocytochemical studies

Human left and right carotid bodies were obtained from twelve cadavers. Briefly, carotid bifurcations were cut out, placed into cold x1 PBS and both carotid bodies were dissected and processed. The left carotid body was directly snap frozen and used for western blotting and right carotid body was immediately fixed in 4% paraformaldehyde and used for immunohistochemistry.

Western blotting: The left human carotid body was crushed in liquid nitrogen with a mortar and pestle. Samples were subsequently homogenised with RIPA Lysis Buffer (Santa Cruz Biotechnology, Inc). Homogenate was centrifuged at 10,000 g for 20 minutes at 4°C and the pellet was discarded. Protein concentration of the supernatant was determined by the Lowry method using the DC-protein assay kit (Bio-Rad Laboratories Ltd). Samples were mixed with loading dye (NuPage, LDS Sample Buffer 4x, Life Technologies) and 20 μg of protein was used for protein electrophoresis on 4-12% Bis-Tris gel (NuPage 4-12% Bis-Tris Gel, Life Technologies). The Amersham ECL Plex Western blotting system using a low-fluorescent PVDF membrane (GE Healthcare, Buckinghamshire, UK) and Alexa Fluor 488 secondary antibody (Life Technologies, UK) were used. This system enables detection and quantification with a broad dynamic range and high linearity. After protein transfer, the membrane was blocked for 1h at room temperature with 2% Advance blocking agent (GE Healthcare). Membranes were incubated with Anti-P2X3 (APR026AN0202; 1:200 dilution) over night at 4°C. The membranes were subsequently washed with 1xPBS/0.1%Tween three times for 10 minutes at room temperature. Incubation with secondary antibody Alexa Fluor 488 was carried out in the dark at room temperature. Before imaging, the membrane was thoroughly washed. Signal was detected by scanning the membrane on a fluorescent

997 laser scanner, Typhoon (GE Healthcare). Protein expressions were quantified using
998 ImageQuant software (GE Healthcare). The specificity of anti-P2X3 antibody was
999 confirmed by membrane incubation with blocking peptide (APR026AG0140, Alomone;
1000 **Supplementary Fig. 10**).
1001
1002 *Immunofluoresce***:** Human right carotid bodies were cut 20 *μm* thick on a cryostat.
1003 Carotid body slices were incubated with 10% goat serum/0.1% Saponin (Sigma-Aldrich,
1004 UK) for 1h at room temperature and gently agitated overnight. Primary antibody
1005 incubation with anti-P2X3 receptor (APR026AN0202, Alomone, Israel; 1:25 dilution)
1006 and anti-tyrosine hydroxylase (TH, F-11, sc-25269, Santa Cruz Biotechnology, Inc;
1007 dilution 1:25) was carried out using manufacturers recommendations in 2% goat serum
1008 over night at 4°C, overnight. Samples were then washed in 1 x PBS four times for 10
1009 minutes at room temperature and the secondary antibody was applied for 1 hour at
1010 room temperature. Anti-rabbit Alexa Fluor 488 was use to visualise the P2X3 receptor
1011 and anti-mouse Alexa Fluor 594 was used to visualise tyrosine hydroxylase. Secondary
1012 antibody incubation was followed by washing as described above and the samples were
1013 mounted on slides using mounting medium (Vectashield, H-1000, Vector Laboratories,
1014 Inc). Carotid bodies were examined under a Leica SP8 AOBS confocal laser scanning
1015 microscope attached to a Leica DM I6000 inverted epifluorescence microscope and
1016 imaging was performed using 'hybrid' GaAsP detectors for greater sensitivity. Images
1017 were processed in Adobe Illustrator CS3.
1018
1019

Methods References

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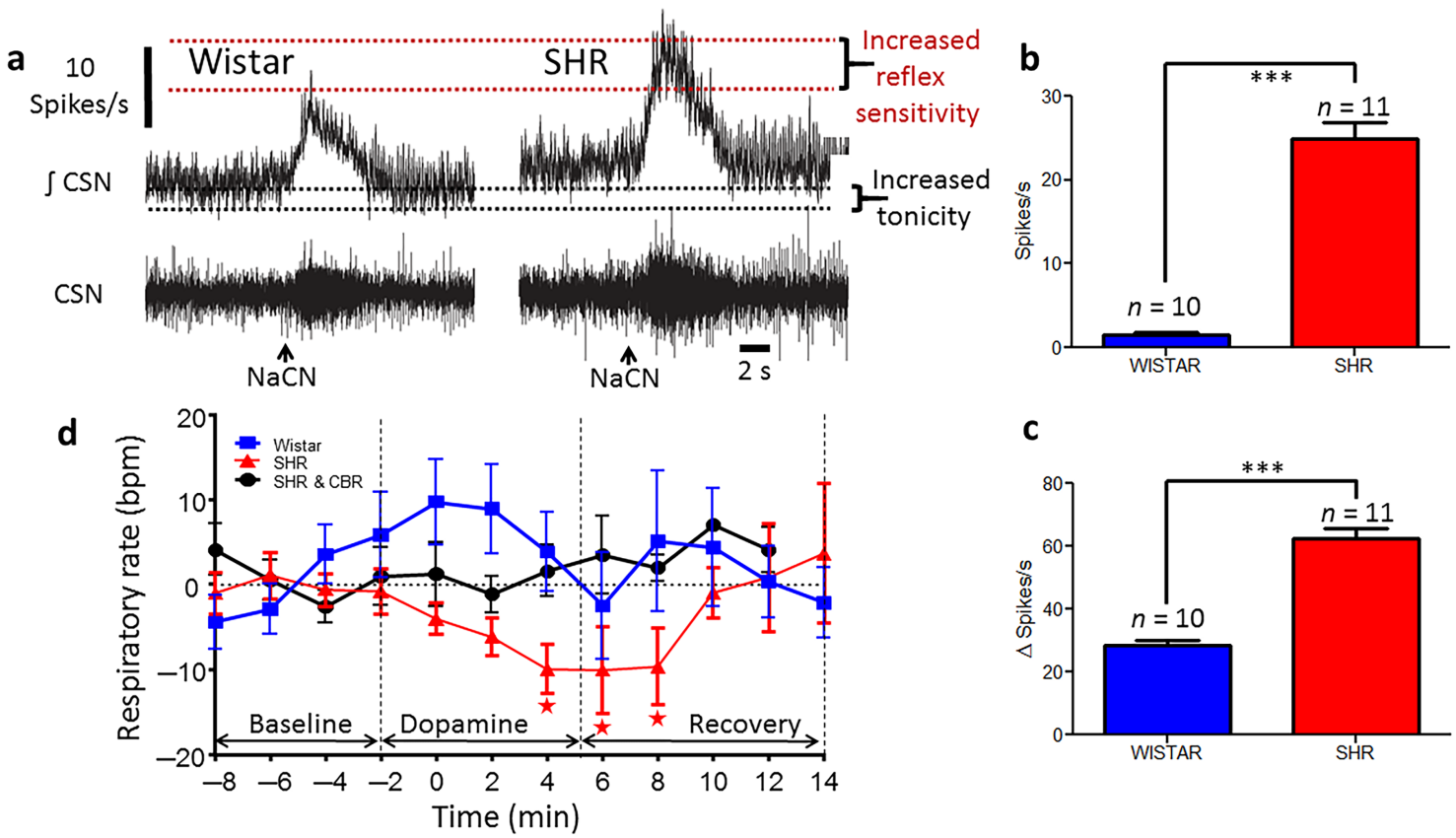
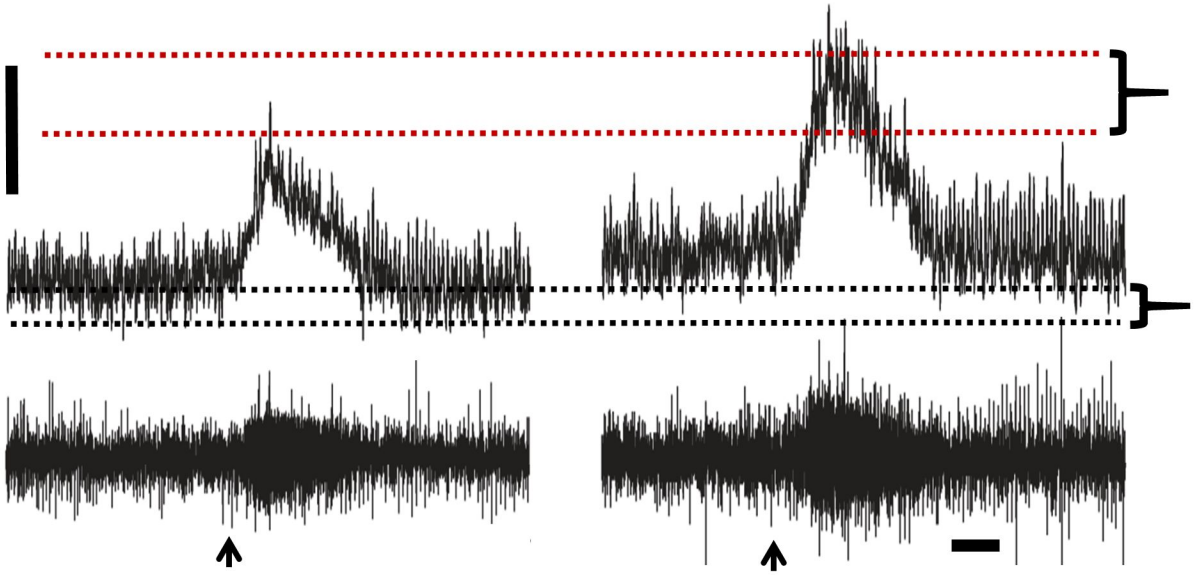
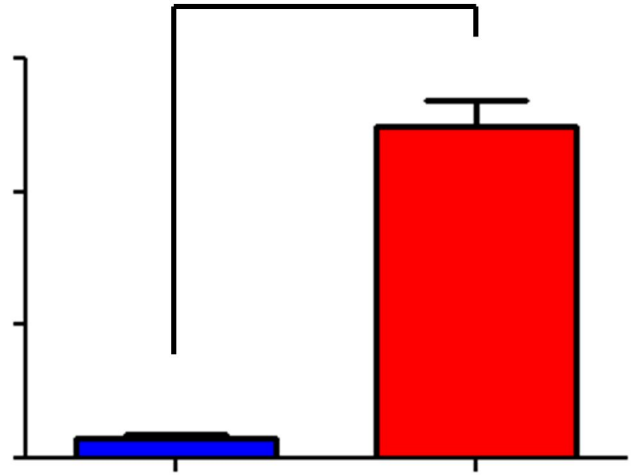
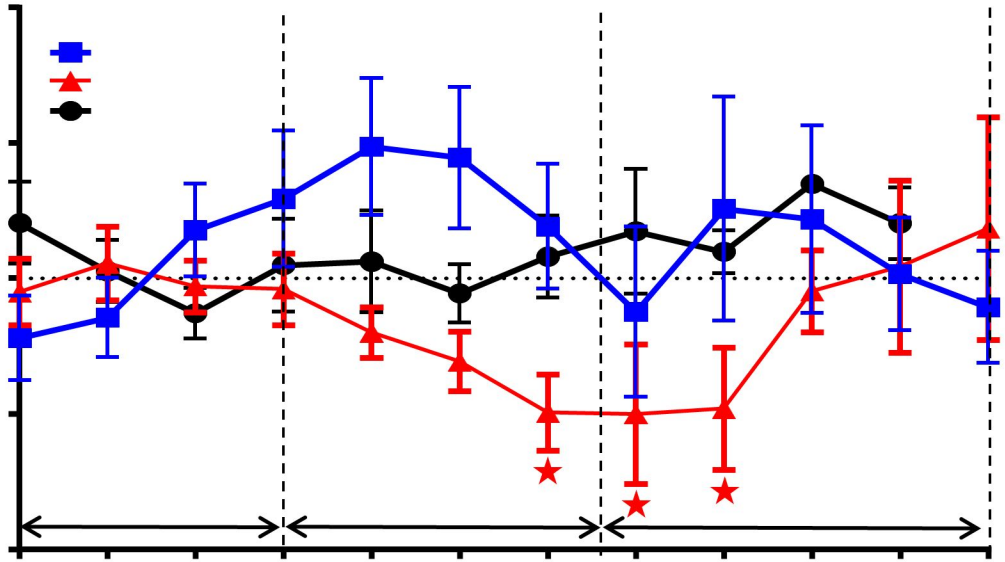
Figure 1


a
10
Spikes/s
Wistar
SHR
Increased
reflex
sensitivity
∫ CSN
Increased
tonicity
CSN
NaCN
NaCN
2 s
b
***
n = 11
n = 10
Spikes/s
WISTAR
SHR
c
***
n = 11
n = 10
Δ Spikes/s
WISTAR
SHR
d
Wistar
SHR
SHR & CBR
Respiratory rate (bpm)
Baseline
Dopamine
Recovery
Time (min)







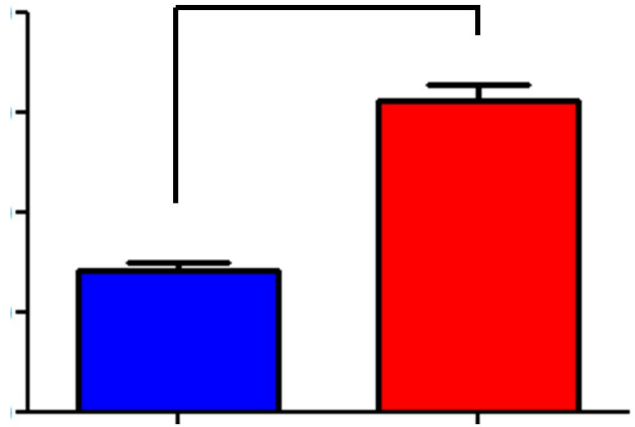


Figure 2

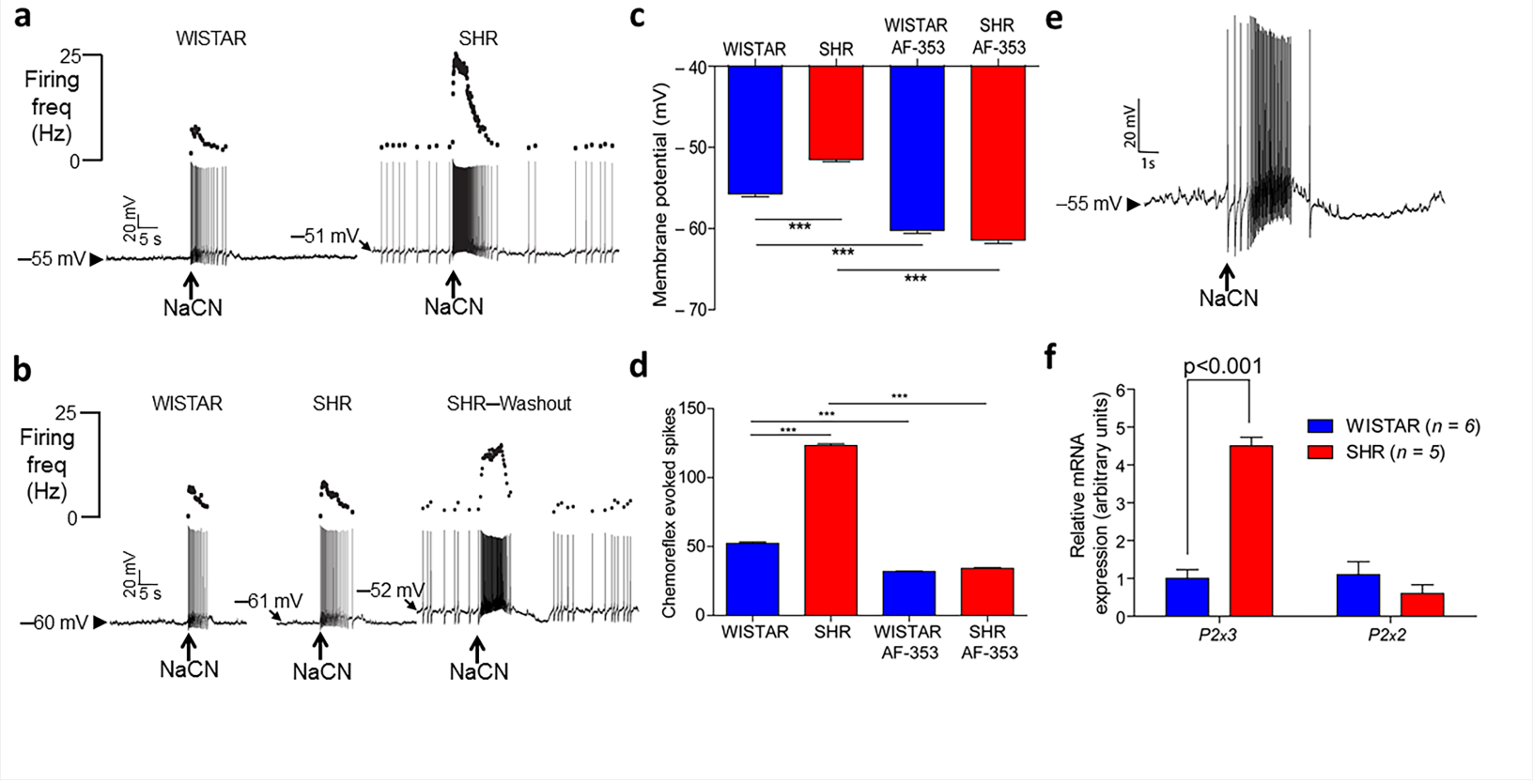

a
WISTAR
SHR
25
Firing freq (Hz)
0
20 mV
5 s
–55 mV
–51 mV
NaCN
NaCN
b
WISTAR
SHR
SHR–Washout
25
Firing freq (Hz)
0
20 mV
5 s
–60 mV
–61 mV
–52 mV
NaCN
NaCN
NaCN
c
WISTAR
SHR
WISTAR AF-353
SHR AF-353
Membrane potential (mV)
–40
–50
–60
–70
***
***
***
d
Chemoreflex evoked spikes
150
100
50
0
***
***
***
WISTAR
SHR
WISTAR AF-353
SHR AF-353
e
20 mV
1s
–55 mV
NaCN
f
p<0.001
Relative mRNA expression (arbitrary units)
6
5
4
3
2
1
0
WISTAR (n = 6)
SHR (n = 5)
P2x3
P2x2

Figure 3

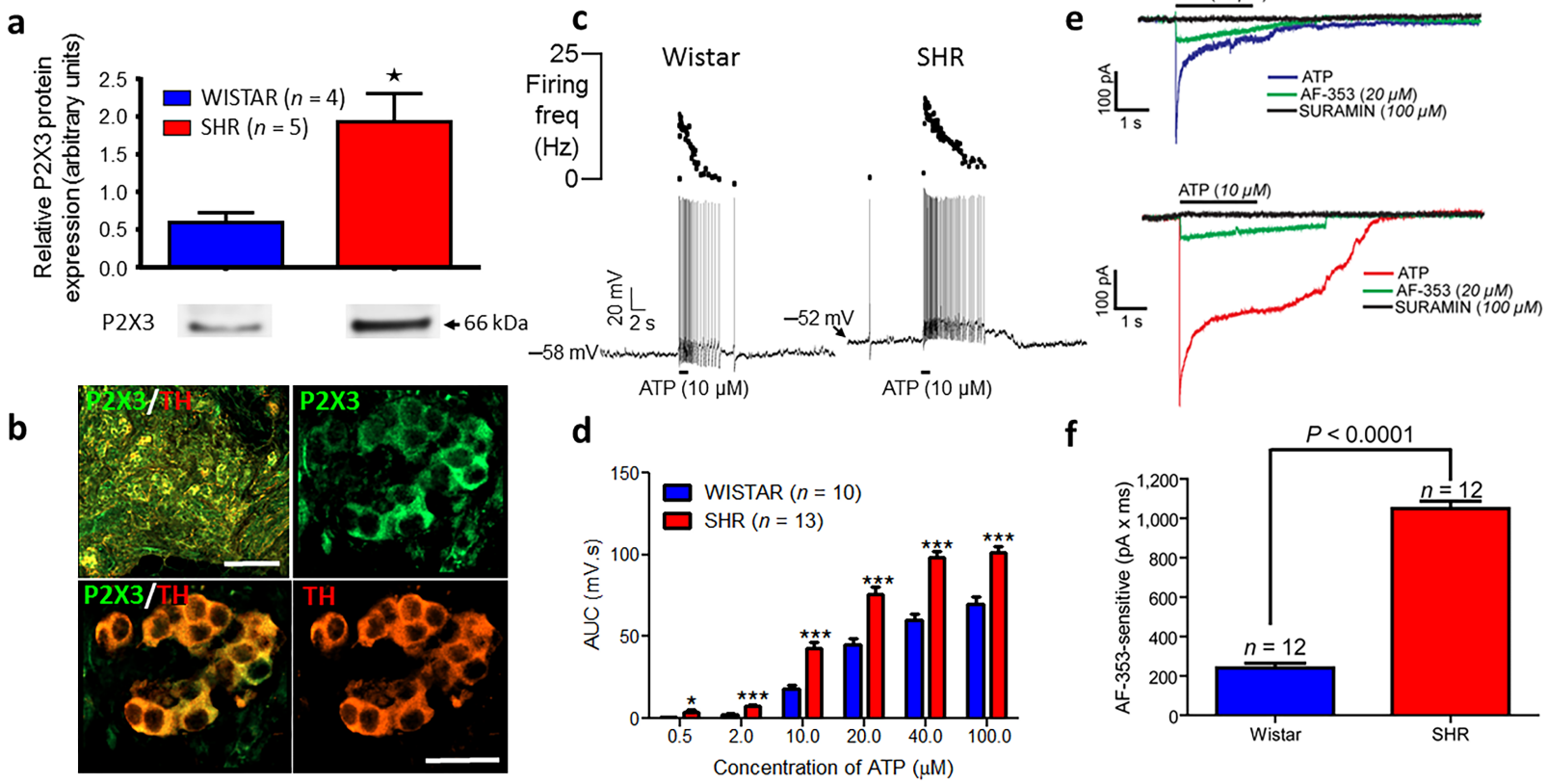

a
Relative P2X3 protein expression (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
WISTAR (n = 4)
SHR (n = 5)
P2X3
66 kDa
b
P2X3/TH
P2X3
P2X3/TH
TH
c
25
Firing freq (Hz)
0
Wistar
SHR
20 mV
2 s
–58 mV
–52 mV
ATP (10 µM)
ATP (10 µM)
d
150
100
50
0
AUC (mV.s)
WISTAR (n = 10)
SHR (n = 13)
*
***
***
***
***
***
0.5
2.0
10.0
20.0
40.0
100.0
Concentration of ATP (µM)
e
ATP (10 µM)
100 pA
1 s
ATP
AF-353 (20 µM)
SURAMIN (100 µM)
ATP (10 µM)
100 pA
1 s
ATP
AF-353 (20 µM)
SURAMIN (100 µM)
f
P < 0.0001
n = 12
n = 12
AF-353-sensitive (pA x ms)
1,200
1,000
800
600
400
200
0
Wistar
SHR

Figure 4

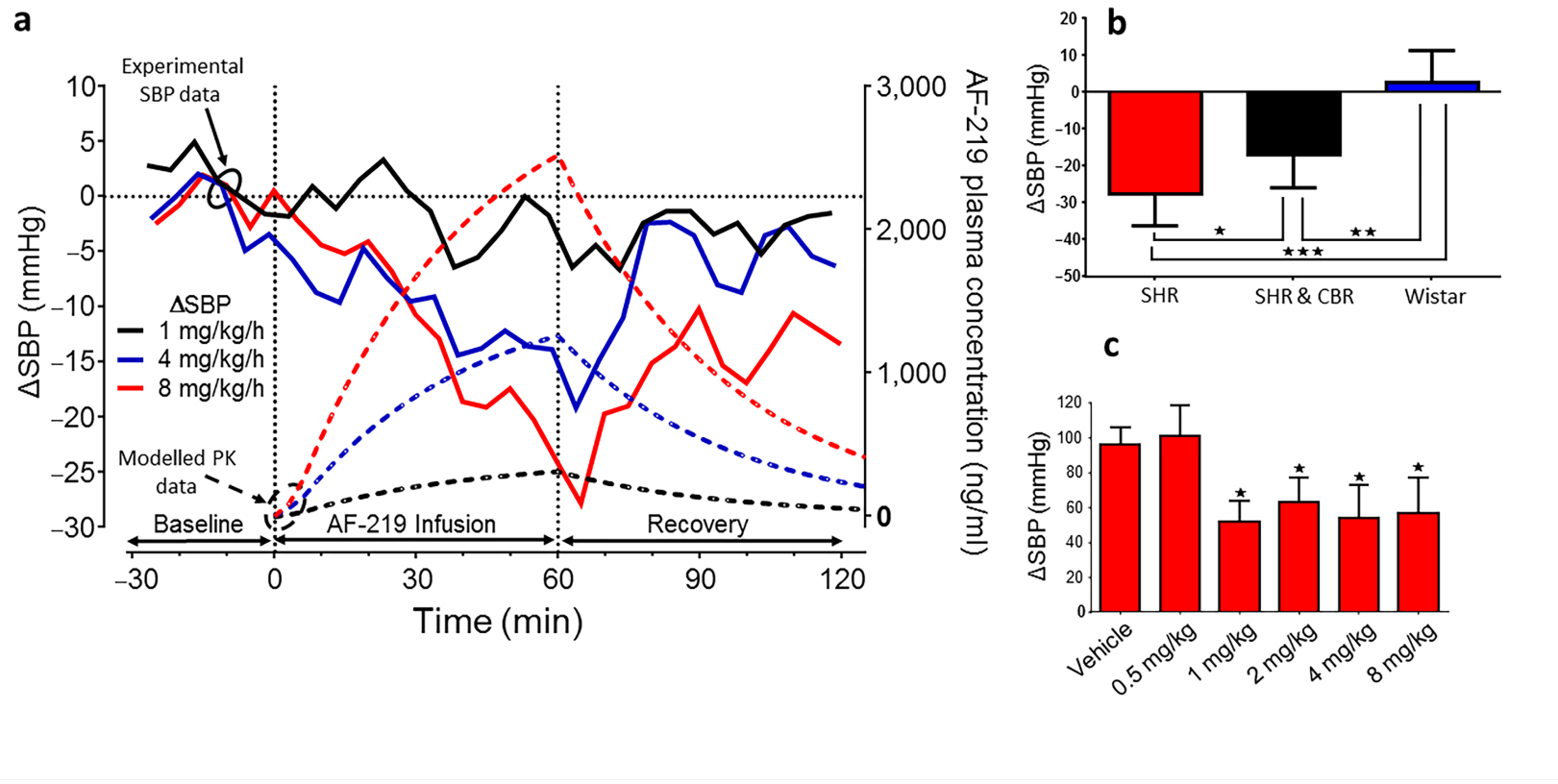

a
Experimental SBP data
ΔSBP (mmHg)
AF-219 plasma concentration (ng/ml)
ΔSBP
1 mg/kg/h
4 mg/kg/h
8 mg/kg/h
Modelled PK data
Baseline
AF-219 Infusion
Recovery
Time (min)
b
ΔSBP (mmHg)
SHR
SHR & CBR
Wistar
c
ΔSBP (mmHg)
Vehicle
0.5 mg/kg
1 mg/kg
2 mg/kg
4 mg/kg
8 mg/kg

Figure 5

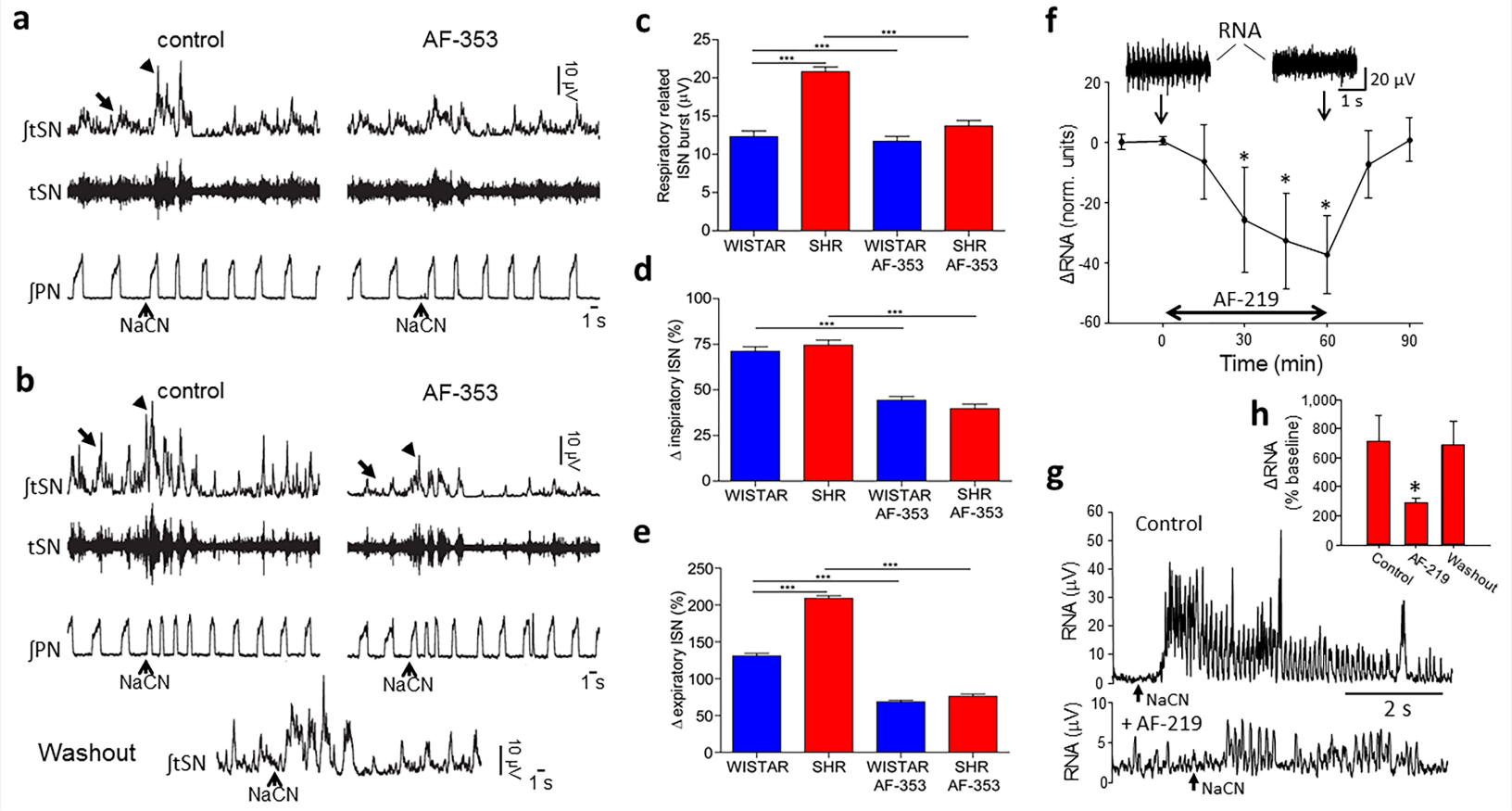
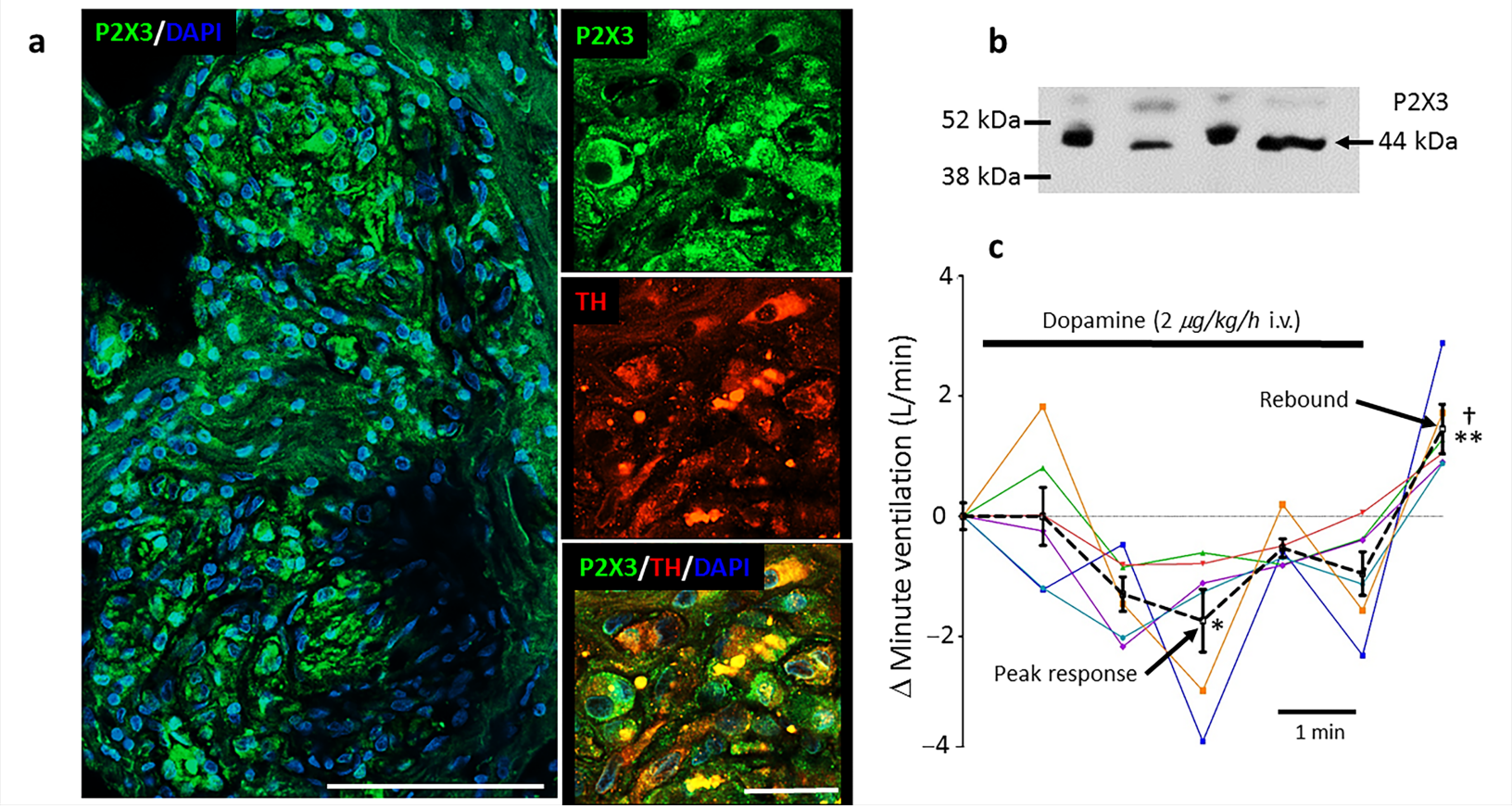

a
control
AF-353
10 µV
∫tSN
tSN
∫PN
NaCN
NaCN
1 s
b
control
AF-353
10 µV
∫tSN
tSN
∫PN
NaCN
NaCN
1 s
Washout
∫tSN
NaCN
10 µV
1 s
c
Respiratory related ISN burst (µV)
25
20
15
10
5
0
***
***
***
WISTAR
SHR
WISTAR AF-353
SHR AF-353
d
Δ inspiratory ISN (%)
100
75
50
25
0
***
***
WISTAR
SHR
WISTAR AF-353
SHR AF-353
e
Δ expiratory ISN (%)
250
200
150
100
50
0
***
***
***
WISTAR
SHR
WISTAR AF-353
SHR AF-353
f
RNA
20 µV
1 s
ΔRNA (norm. units)
20
0
-20
-40
-60
*
*
*
AF-219
0
30
60
90
Time (min)
g
Control
RNA (µV)
60
50
40
30
20
10
0
NaCN
2 s
+ AF-219
RNA (µV)
10
5
0
NaCN
h
ΔRNA (% baseline)
1,000
800
600
400
200
0
*
Control
AF-219
Washout

Figure 6


a
P2X3/DAPI
P2X3
TH
P2X3/TH/DAPI
b
52 kDa
38 kDa
P2X3
44 kDa
c
Dopamine (2 μg/kg/h i.v.)
Rebound
Peak response
1 min
Δ Minute ventilation (L/min)
4
2
0
−2
−4
*
**
†